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(54) Inositoltriphosphate, a method for preparing same and a composition containing same.

(57) An inositoltriphosphate (IP_3) selected from the group consisting of D-myo-inositol-1.2.6-triphosphate, D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate, a method for producing the same and a composition comprised of the same.

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Inositoltriphosphate, a method for preparing same and a composition containing same

The present invention relates to a special inositoltriphosphate (IP_3), a method of producing the same and a composition containing the same.

Even as early as the year 1900, different researchers had reported the finding of the organic phosphate compound phytic acid, i.e., 1,2,3,4,5,6-hexakis (dihydrogenphosphate) myo-inositol (also sometimes called inositol-hexaphosphoric acid) in plants. The content of phytic acid in different plants varies considerably. The content in grain is usually approximately 0.5-2%, with certain exceptions. Polished rice has a level of only 0.1% while wild rice contains as much 2.2% phytic acid. Beans contain about 0.4-2%, oil plants approximately 2-5% and pollen 0.3-2% The content of phytic acid in the plant varies during the growth period. The content is also influenced by, among other things, the climate.

In the literature there are reports on the presence of inositol pentaphosphate (IP_5) and inositol tetraphosphate (IP_4) in a few plants. It is further known that phosphate derivates lower than IP_6 are formed at germination of grain. For instance the final products at the germination are inositol and phosphate. The use of IP_6 has been described in several scientific publications. The majority of the authors of these articles have observed several negative effects on humans and animals when consuming IP_6 or substances containing IP_6. Feeding dogs with too high an amount of IP_6 gives rise for example to rachitis. In humans lack of zinc and as a consequence thereof slower growth of children has been observed. Anemia has been observed mainly in women. Because of the above mentioned negative effects on the mineral balance in humans and animals, attempts have so far been made to reduce the intake of IP_6 and its derivatives to a minimum.

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Furthermore, it is known for instance from Bull. Sté. Chim. Biol. 36,9 (1956) p. 85 to hydrolyse phytic acid with diluted hydrochloric acid at an increased temperature to obtain a mixture of lower inositolphosphates, i.e. IP_5, IP_4, IP_3, IP_2 (inositoldiphosphate) and IP_1 (inositolmonophosphate). Each of these inositolphosphates can be present in the form of many isomers. Up to 20 isomers can be expected for IP_3.

One specific isomer of IP_3, i.e. D-myo-inositol-1.4.5-triphosphate has been reported in Biochem. Biophys. Res. Commun. 120,2 (1984), page 481. This compound is known as an intracellular calcium mobilizer in the human body and it can readily be isolated from cell membranes.

Nothing is known about the properties of any other of the specific isomers of the different inositoltriphosphates in pure form. Thus, it is so difficult to separate the large number of IP_3 isomers from each other, thereby identify and define the structural formula of each isomer and its properties. Up to the present there is no known method for producing or obtaining any single isomer of IP_3 other than the aforementioned D-myo-inositol-1.4.5.-triphosphate. Further in a process for the production of IP_3 which includes a hydrolytic system, a re-arrangement of the isomers and/or a further dephosphorylation to IP_2, IP_1 or inositol must be considered as special problems.

Due to above difficulties there are no data on specific IP_3 isomers in substantially pure form other than the above-mentioned D-myo-inositol-1.4.5-triphosphate.

According to the present invention it has quite unexpectedly been possible to solve the above problem of separating certain different isomers of IP_3 from each other and produce them in substantially pure form. The IP_3 isomers can be obtained as a salt or as an acid thereof. The salt form is preferred, since it is easier to produce in pure and concentrated form than the acid.

According to the invention an inositoltriphosphate (IP_3) selected from the group consisting of D-myo-inoistol-1.2.6-triphosphate, D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate in either acid or salt form has been produced and isolated in substantially pure form.

As mentioned above the individual IP_3 isomer can be obtained as a salt or an acid thereof. In both forms it can for the first time be obtained in substantially pure form.

The IP_3 in acid form is generally provided in the form of an aqueous solution. In such a case the concentration of the acid may be from 10 to 45, preferably 15 to 45 or most preferably 20 to 45 % by weight of the total weight of the solution.

The salt form of the IP_3 isomer is readily obtainable from the acid form using standard procedures. Thus, there can be prepared salts such as alkali metal and alkaline earth metal salts e.g. lithium, sodium, potassium, calcium or magnesium. However, also the aluminium, zinc and iron salts are very useful as well as the NH_4^+ and organic amine salts. Moreover, mixed salts containing different cations can be used.

Exemplary amines are triethanolamine, diethanolamine, triisopropanolamine, N,N-dimethyl-2-amino-2-methyl-1-propanol, N,N-dimethyl-ethanolamine, tetrabutylamine and cyclohexylamine. Also other salts might be useful. Especially preferred are salts which are physiologically acceptable.

For production of the isomer or isomers of IP_3 according to the invention, one or more of the compounds IP_6, IP_5 or IP_4 or a natural product containing at least one of these compounds can be used as a starting material. It is preferred to use IP_6 containing materials, since they are most easily available. In the cases where the starting material is a natural product, one with a content of at least 0.3 %, preferably at least 1 % by weight of inositolphosphate ($IP_6 + IP_5 + IP_4$) is preferably chosen.

Especially suitable products are grain, particularily bran, pollen, beans and oil plants. These products all contain IP_6.

In theory the IP_3 isomers are believed to be producible by for example the following techniques.

1) enzymatic breakdown starting from IP_4, IP_5 and/or IP_6.
2) chemical hydrolysis starting from IP_4, IP_5 and/or IP_6.
3) chemical synthesis starting, for example, with inositol, IP_1, IP_2 and phosphate.
4) enzymatic synthesis starting, for example, with inositol, IP_1, IP_2 and phosphate.
5) microbiological production (including also hybrid DNA-techniques).
6) chemical or enzymatic migration of inositolphosphate or chemical or enzymatic hydrolysis of substituted inositol-phosphate.

Combination of two or more of the above mentioned procedures may also be possible. However many of these procedures produce only mixtures of a number of isomers which are at best extremely difficult to separate to individual isomers, if separable at all.

According to the invention a procedure where a material containing IP_6 is used is preferred as mentioned before. Then IP_6 is broken down enzymatically to IP_3 with phytase enzyme. Phytase enzyme is normally present in all inositolphosphate containing plants and seeds. Because of this it is, according to the invention, usually not necessary to add the enzyme if a natural product is used as starting material. In the cases where the natural product has too low an enzymatic activity or when IP_6, IP_5 or IP_4 or a mixture of these is used as starting material, a phytase enzyme from bran, for example, is added.

Phytase enzyme from plants, seeds and microorganisms has the surprising effect to make it possible, according to the invention, to produce the specific IP_3 isomers mentioned above in high concentration and in substantially pure form.

The IP_6 can be provided either as pure material or in the form of an IP_6 containing source. A suitable way to treat a natural starting material containing IP_6, e.g. bran is to pre-treat it, for instance by breakage or removal of outer membrane and removal of unwanted constituents. Thereafter the material is soaked in water to make the inositolphosphate available for breaking down and to activate the enzyme. Where additional enzyme is necessary, this may be added at this stage or a later stage. The enzyme is then allowed to act for so long a time as is necessary for the intended degree of hydrolysis to be achieved.

The hydrolysis takes place at a suitable temperature, usually 20-70°C, preferably 30-60°C and at a pH of 4 to 8. In order to stop the hydrolysis at the intended level the enzyme may be destroyed or inactivated, for instance by a rapid heating of the hydrolysed starting material. In order to transfer the material to a form which is stable at storage it can suitably be freeze dried.

The invention especially relates to a method of producing an inositoltriphosphate (IP_3) selected from the group consisting of D-myo-inositol-1.2.6-triphosphate, D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate in either acid or salt form, wherein an IP_6 containing material is incubated at a temperature ranging between 20 and 70°C, preferably 30 to 50°C and a pH of 4 to 8 with phytase until the liberation of about 30-60% usually about 50% of the total ester phosphorus has been achieved. At said stage a high proportion of the desired IP_3 isomer or isomers has been formed by hydrolysis of the IP_6 containing material.

The mixture of inositolphosphates obtained may hereafter be separated by column chromatography to isolate the IP_3-containing fraction. In case of chromatographic separation the said fraction is then optionally subjected to another chromatographic separation, preferably in a column. Such a separation may offer advantages if the fraction contains more than one IP_3 isomer. The IP_3 isomer or isomers are then preferably isolated in acid form. By adding a base to the acid, the IP_3 in salt form can be obtained, if desired.

Among the many sources of phytase useful according to this invention, yeast and most preferably baker's yeast is preferred. Swedish baker's yeast produced by Jästbolaget, Sweden as well as baker's yeast produced by Rajamäki Finland and Hefefabriken AG, Switzerland have for instance been used according to the present invention. When using such yeast it has been established very surprisingly that essentially only one isomer is obtained, namely D-myo-inositol-1.2.6-triphosphate. Of course, the use of yeast is a very valuable procedure when the said isomer only is desirable.

In accordance with present knowledge no other method will provide a single isomeric product. Usually a mixture of a lot of isomers is obtained.

The above mentioned procedure, with appropriate modifications, can be used also when one or more of the compounds IP_6, IP_5 or IP_4 per se are used as starting material.

In another embodiment the invention also relates to an inositolphosphate composition comprised of D-myo-inositol-1.2.6-triphosphate, and at least one of D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate in acid or salt form. The composition usually contains 20-99.5, preferably 30-99.5 % by weight of said IP_3. The content of D-myo-inositol-1.2.6-triphosphate is in the range from 50-100 %, by weight based on the total content of said inositoltriphosphates, the balance including inositolphosphates other than IP_3.

Sometimes it might be preferable that the IP_3 in the composition consists essentially of D-myo-inositol-1.2.6-triphosphate solely.

In addition to IP_3 the balance of the inositolphosphate composition can contain also inositoltetraphosphate (IP_4) and inositoldiphosphate (IP_2).

The composition can consist of 20-99.5 %, preferably more than 60 %, by weight of IP_3 and 80-0.5 %, preferably less than 40%, by weight of other inositolphosphates. 40-85 %, preferably 50-85 %, by weight of said other inositolphosphates should then consist of IP_2 plus IP_4. It may be preferable that the IP_3 consists essentially of D-myo-inositol-1.2.6-triphosphate.

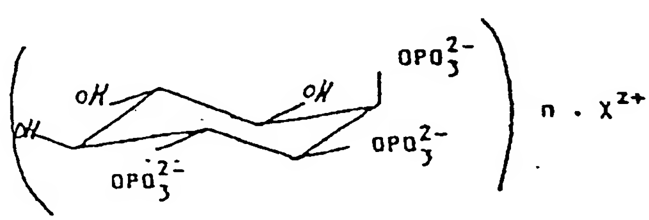
The composition sometimes contains minor amounts, e.g. less than 10 %, preferably 1-8 % by weight of one or more of IP_1, and IP_6 calculated on the total content of inositolphosphates in the composition.

Such type of compositions are preparable by enriching the initial fermentation inositolphosphate product by addition of the D-myo-inositol-1.2.6-triphosphate to the desired final concentration of the said isomer in the composition. Such methods are well-known in the art and include e.g. simple mechanical mixing. Alternatively this composition may be produced directly by fermentation using yeast, especially baker's yeast as the phytase source. As previously indicated the use of baker's yeast results in the almost exclusive production of D-myo-inositol-1.2.6-triphosphate, i.e. substantially all of the IP_3 fraction is D-myo-inositol-1.2.6-triphosphate.

The inositoltriphosphate in acid or salt form according to the invention can be used as a pharmaceutical or foodstuff, optionally in additive form or as a stabilizer for various products. Moreover, IP_3 can give a protecting effect on seed. It can also be used as an additive for tooth-paste, as a corrosion inhibitor in paint, lacquers, lubricating oils and at surface treatment of metals, as a component in a cleansing agent, as a flame-resistant agent, for lithographic applications, for inhibition of e.g. aflatoxin production in microorganisms and for a modification or increase of the enzyme activity of amylase, for instance.

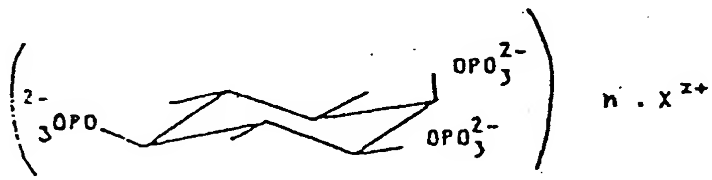
The inositol-phosphate composition produced either directly by fermentation or by enrichment as previously described is useful in all of the afore mentioned applications.

The IP_3-isomers mentioned above have the following formulas: D-myo-inotitol-1.2.6-triphosphate of the formula

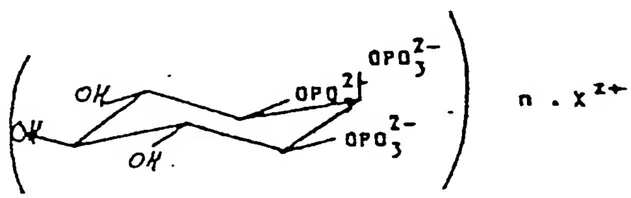


where X is hydrogen, at least one univalent, divalent or multivalent cation, or a mixture thereof, n is the number of ions, and z is the charge of the respectively ion;

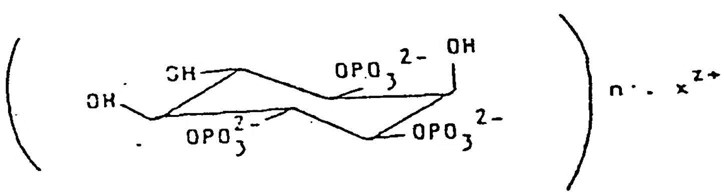
D-myo-inositol-1,2,5-triphosphate of the formula



where X, n and z have the above mentioned meaning; myo-inositol-1.2.3-triphosphate of the formula



where X, n and z have the above mentioned meaning; and L-myo-inositol-1.3.4-triphosphate of the formula



where X, n and z have the above mentioned meaning.

In each of the above formulas, n ranges between 6 to 1 inclusive and z ranges from 1 to 6 inclusive. Preferably, n is between 3 to 6 inclusive and z is 3, 2 or 1.

The new IP_3 isomer of the present invention are particular-ily effective in the afore said therapeutic use and they are especially devoid of any undesirable side-effects in this use. In particular, D-myo-inositol-1.2.6-triphosphate is especially effective and demonstrates a higher order of activity in comparison with the other isomers, especially D-myo-inositol-1.4.5-triphosphate. Complexes formed by D-myo-inositol-1.2.6-triphosphate with Cd are considerably more stable than Cd complexes formed with for example D-myo-inositol-1.4.5-triphosphate. To a lesser degree D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate are also more desirable for therapeutic use than D-myo-inositol-1.4.5-triphosphate for many of the same reasons.

The invention will be explained further in connection with the embodiment examples below and the enclosed figures and table. Examples 1 and 2 show a hydrolysis of sodium phytate with wheat phytase and fractionation of a mixture of inositol-phosphates. Examples 3 and 4 relate to structural determination of isomers of IP_3. Example 5 illustrates a determination of pKa-values for IP_3. Example 6 shows a determination of relative binding constants for IP_3 with Ca, Zn and Cd respectively. Examples 7-10 relate to work-up procedures for calcium salts of the IP_3-isomers of the invention. Example 11 illustrates the infrared spectrum of the calcium salt of D-myo-inositol-1.2.6-triphosphates. Examples 12 and 13 show a hydrolysis of sodium phytate with vheat bran and a fractionation of the mixture of inostitolphosphates obtained. Example 14 relates to a work-up procedure of the zinc salt of D-myo-inositol-1.2.6-triphosphate. Examples 15-17 show a hydrolysis of sodium phytate with baker's yeast, a fractionation of the mixture of inositolphosphates obtained and a determination of the sole isomer of IP_3 obtained.

Example 18 illustrates a work-up procedure of the sodium salt of D-myo-inositol-1.2.6-triphosphate. Example 19 shows a chemical hydrolysis of sodium phytate with hydrochloric acid and a fractionation of the mixture of inositolphosphates obtained. Example 21 relates to a chemical synthesis of inositolphosphates from polyphosphoric acid and myo-inositol and a structural determination of the IP_3 with H-NMR. Example 21 shows hydrolysis of phytic acid in rice bran, extraction and analyzis of the inositolphosphates obtained. Example 22 relates to a characterization of different salts of D-myo-inositol-1.2.6-triphosphate. Example 23 shows that IP_3 prevents an increase of platelet aggregation in humans caused by smoking. In example 24 it is shown that an increased blood glucose level in mice caused by free radicals can be counteracted by injection of IP_3.

Example 1

Hydrolysis of sodium phytate with wheat phytase and fractionation of a mixture of inositolphosphates.

A 1.6 gram quantity of sodium phytate (from corn, Sigma Chemical Co, St. Louis, Missouri, USA) was dissolved in 650 ml sodium acetate buffer, pH 5.2. 2.7 gram wheat phytase (EC 3.1.3.26; 0.015 U/mg, from Sigma Chemical Co) was added and the mixture was incubated at 38^{o}C.

The dephosphorylation was followed by determining the inorganic phosphorus released. After 3 hours when 50% inorganic phosphorus had been liberated the hydrolysis was stopped by adding 30 ml ammonia to pH 12. A liquid mixture containing inositolphosphates was obtained.

350 ml of the mixture was passed through an ion-exchange column (Dowex 1, chloride form, 25 mm x 250 mm) and eluted with a linear gradient of hydrochloric acid (0-0.7 N HCl). Aliquots of eluted fractions were completely hydrolyzed in order to determine the contents of phosphorus and inositol. The amount of phosphorus versus eluted volume is shown in fig 1. The peaks correspond to different inositolphosphates i.e. a peak with the ratio of phosphorus to inositol of three to one consists of inositoltriphosphate etc. Two fractions with the ratio of phosphorus to inositol of three to one were obtained.

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Example 2

Fractionation of inositoltriphosphates.

100 ml of the first fraction obtained in Example 1 with a phosphorus/inositol ratio of three to one was neutralized and precipitated as a bariumsalt after addition of 10 % excess of 0.1 M bariumacetate solution. 600 mg of the precipitated salt was dissolved in 50 ml diluted hydrochloric acid. The solution was separated on an ion-exchange column (Dowex 1, chloride form, 25 mm x 2500 mm) with diluted hydrochloric acid as eluent. Aliquots of eluted fractions were analyzed for phosphorus. The amount of phosphorus versus eluted volume is shown in fig 2. Three peaks consisting of isomers of inositoltriphosphates can be seen in the figure.

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Example 3

Structural determination of isomers of inositoltriphosphates with H-NMR.

The three peaks obtained in Example 2 were analyzed by H-NMR. The spectra are shown in fig. 3 a, b and c. Data show that the peaks consist of myo-inositol-1.2.6-triphosphate, myo-inositol-1.2.3-triphosphate and myo-inositol-1.3.4-triphosphate respectively.

The second fraction obtained in Example 1 with a phosphorous/inositol ratio of three to one was analyzed by H-NMR. The spectrum is shown in fig. 4. Data show that the fraction consists of myo-inositol-1.2.5-triphosphate. In this embodiment example as well as in all the following ones where H-NMR was used, the H-NMR instrument was a Nicolet 360 WB spectrometer. The internal standard was tetramethylsilane.

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Example 4

Determination of optical isomers of inositoltriphosphates.

20 mg of the compounds determined with H-NMR according to Example 3 to be myo-inositol-1.2.6-triphosphate and myo- inositol-1.3.4-triphosphate were further chromatographed on a chiral column based on acetylated cellulose (20 mm x 300 mm from Merck) with a mixture of ethanol and water as eluent. The fractions were analyzed with a polarimeter. As can be seen in fig 5 each compound consists of one optical isomer, D-myo-inositol-1.2.6-triphosphate and L-myo-inositol-1.3.4-triphosphate respectively.

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Example 5

Determination of pKa-values for inositoltriphosphates

A 10 ml quantity of the first fraction obtained in Example 1 with a phosphorus/inositol ratio of three to one was titrated with 0.01 M NaOH. The pH during the titration was measured with an electrode. Fig 6 shows the pH versus volume NaOH.

The following pKa-values were obtained:

pKa1 = 4.7
pKa2 = 7.5

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Example 6

Determination of relative binding constants for inositol-triphosphates and calcium, zinc and cadmium respectively.

A 10 ml quantity of the first fraction obtained in Example 1 with a phosphorus/inositol ratio of three to one was titrated with 0.01 M NaOH in the presence of 0.2 mM calcium, zinc and cadmiumions respectively. A greater tendency to formation of an inositoltriphosphate-metalcomplex results in lowering the pH at a certain volume NaOH added. As can be seen in fig 7 the metal binding constants for IP_3 increase in the following order:

Ca < Zn < Cd

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Example 7

Work-up procedure of calciumsalt of D-myo-inositol-1.2.6-triphosphate.

100 ml of the fraction containing D-myo-inositol-1.2.6-triphosphate obtained in Example 2 was neutralized to a pH of about 7 with an aqueous solution of $Ca(OH)_2$. The calciumsalt was precipitated by the addition of 100 ml ethanol. The precipitate was centrifuged recrystallized and dried in vacuum.

The purified calciumsalt of D-myo-inositol-1.2.6-triphosphate obtained was structurally confirmed by analysis with H-NMR.

The above recrystallized calciumsalt of D-myo-inositol-1.2.6-triphosphate was also chemically analyzed in order to determine the content of carbon phosphorus, oxygen and calcium. Table 1 shows the result. The formula of the salt is Ca_3IP_3.

Example 8

Work-up procedure of calciumsalt of L-myo-inositol-1.3.4-triphosphate.

100 ml of the fraction containing L-myo-inositol-1.3.4-triphosphate obtained in Example 2 was neutralized to a pH of about 7 with an aqueous solution of $Ca(OH)_2$. The calciumsalt was precipitated by the addition of 100 ml ethanol. The precipitate was centrifuged, recrystallized and dried in vacuum.

The purified calciumsalt of L-myo-inositol-1.3.4-triphosphate was structurally confirmed by analysis with H-NMR.

The above recrystallized calciumsalt of L-myo-inositol-1.3.4-triphosphate was also chemically analyzed in order to determine the content of carbon phosphorus, oxygen and calcium. Table 1 shows the result. The formula of the salt is Ca_3IP_3.

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Example 9

Work-up procedure of calciumsalt of myo-inositol-1.2.3-triphosphate.

100 ml of the fraction containing myo-inositol-1.2.3-triphosphate obtained in Example 2 was neutralized to a pH of about 7 with an aqueous solution of $Ca(OH)_2$. The calciumsalt was precipitated by the addition of 100 ml ethanol. The precipitate was recrystallized and dried in vacuum.

The purified calciumsalt of myo-inositol-1.2.3- triphosphate was structurally confirmed by anlysis with H-NMR.

The above recrystallized calciumsalt of myo-inositol-1.2.3-triphosphate was also chemically analyzed in order to determine the content of carbon, phosphorus, oxygen and calcium. Table 1 shows the result. The formula of the salt is Ca_3IP_3.

Example 10

Work-up procedure of calciumsalt of D-myo-inositol-1.2.5-triphosphate.

100 ml of the fraction containing D-myo-inositol-1.2.5-triphosphate obtained in Example 1 was neutralized to a pH of about 7 with an aqueous solution of $Ca(OH)_2$. The calciumsalt was precipitated by the addition of 100 ml ethanol. The precipitate was centrifuged, recrystallized and dried in vacuum.

The purified calciumsalt of D-myo-inositol-1.2.5-triphosphate was structurally confirmed by analysis with H-NMR.

The above recrystallized calciumsalt of D-myo-inositol-1.2.5-triphosphate was also chemically analyzed in order to detemine the content of carbon, phosphorus, oxygen and calcium. Table 1 shows the result. The formula of the salt is Ca_3IP_3.

Example 11

Infrared (IR) spectrum of the calciumsalt of D-myo-inositol-1.2.6-triphosphate.

The purified calciumsalt of D-myo-inositol-1.2.6-triphosphate obtained in Example 7 was analyzed with IR. The characteristic bands are:

3500 cm^{-1}	-	OH
2900 cm^{-1}	-	CH
1600 cm^{-1}	-	OH
1100 cm^{-1}	-	C-O and -P
1000 cm^{-1}	-	C-O and -P
800 cm^{-1}	-	C-C

Example 12

Hydrolysis of sodium phytate with wheat bran and fractionation of a mixture of inositolphosphates.

A 10 gram quantity of sodium phytate (from corn Sigma Chemical Co) was dissolved in 500 ml sodium acetate, buffer at pH 5.0. With the temperature increased to 37°C, wheat bran (10 g) was added at stirring. Incubation was started and continued at 37°C. The dephosphorylation was followed by determining the inorganic phosphorus released. The hydrolysis was stopped by addition of 100 ml ammonia after 2 hours when 50 % inorganic phosphorus had been liberated. The suspension obtained was centrifuged and the supernatant was collected.

300 ml of the supernatant was passed through an ion-exchange column (Dowex 1, chloride form, 25 mm x 250 mm) and eluted with a linear gradient of hydrochloric acid (0-0.7 N HCl). Aliquots of eluted fractions were completely hydrolyzed in order to detemine the contents of phosphorus and inositol. Two fractions with the phosphorus/inositol ratio of three to one (IP_3) were collected.

Example 13

Fractionation of inositoltriphosphates

The same method was used as described in Example 2 except for the difference that the first fraction collected in Example 12 was chromatographed. Three peaks were obtained and analyzed by H-NMR. The peaks consist of myo-inositol-1.2.6-triphosphate, myo-inositol-1.2.3-triphosphate and myo-inositol-1.3.4-triphosphate respectively.

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Example 14

Work-up procedure of the zincsalt of D-myo-inositol-1.2.6-triphosphate.

100 ml of the fraction containing D-myo-inositol-1.2.6-triphosphate obtained in Example 13 was neutrilized to a pH of about 7 with an aqueous solution of ZnO. The zincsalt was precipitated at the addition of 100 ml ethanol. The precipitate was centrifuged, recrystallized and dried in vacuum. The above recrystallized zincsalt of D-myo-inostiol-1.2.6-triphosphate was also chemically analyzed in order to determine the content of carbon, phosphorus, oxygen and zinc. Table 1 shows the result. The formula of the salt is Zn_3IP_3.

Example 15

Hydrolysis of sodium phytate with baker's yeast and fractionation of a mixture of inositolphosphates.

A 0.7 gram quantity of sodium phytate (from corn, Sigma Chemical Co) was dissolved in 600 ml sodium acetate buffer at pH 4.6. 50 gram of baker's yeast from Jästbolaget, Sweden (solid contents 28 %, nitrogen content 2 %, phosphorus content 0.4 %) was added at stirring. Incubation was started and continued at 45°C. The dephosphorylation was followed by determining the inorganic phosphorus released. After 7 hours when 50% inorganic phosphorus had been liberated the hydrolysis was stopped by adding 30 ml of ammonia to pH 12. The suspension was centrifuged and the supernatant was collected.

400 ml of the supernatant was passed through an ion-exchange column (Dowex 1, chloride from, 25 mm x 250 mm) and eluted with a linear gradient of hydrochloric acid (0-0.7 N HCl).

Aliquots of eluted fractions were completely hydrolyzed in order to determine the contents of phosphorus and inositol. The amount of phosphorus versus eluted volume, is shown in fig 8. The peaks correspond to different inositolphosphates i.e. a peak with the ratio of phosphorus to inositol of three to one consists of inositol-triphosphates etc.

Example 16

Structural determination of isomers of inositoltriphosphate.

The fraction obtained in Example 15 with a phosphorus/ inositol ratio of three to one was neutralized and evaporated before analysis with H-NMR. The spectrum proved to be identical with that shown in fig 3a. Data show that the peak consists of myo-inositol1.2.6-triphosphate.

Example 17

Determination of optical isomers of myo-inositol-triphosphate.

The same method was used as described in Example 4 with the difference that 10 mg of the compound determined with NMR according to Example 16 was analyzed. As can be seen in fig 9 the compound consists of one optical isomer, D-myo-inositol-1.2.6-triphosphate. Said isomer can be rearranged to L-myo-inositol-1.3.4-triphosphate by treatment with an acid such as hydrochloric acid.

Example 18

Work-up procedure of the sodiumsalt of D-myo-inositol-1.2.6-triphosphate.

100 ml of the fraction containing D-myo-inositol-1.2.6-triphosphate obtained in Example 15 was neutralized to a pH of about 7 with an aqueous solution of NaOH. After addition of 100 ml ethanol the volume of the solution was reduced by evaporation and the sodiumsalt was precipitated, centrifuged, recrystallized and dried in vacuum. The purified sodiumsalt of D-myo-inositol-1.2.6-triphosphate obtained was structurally confirmed by analysis with H-NMR.

The recrystallized sodiumsalt of D-myo-inositol-1.2.6-triphosphate was chemically analyzed in order to determine the contents of carbon, phosphorus, oxygen and sodium. Table 1 shows the result. The formula of the salt is Na_6IP_3.

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Example 19

Chemical hydrolysis of sodium phytate and fractionation of a mixture of inositolphosphates.

A 1.0 gram quantity of sodium phytate (from corn, Sigma Chemical Co) was dissolved in 15 ml 6 N HCl. The sample was heated under vacuum in a sealed tube in an oven (105^oC) for 5 hours. After this time 34 % of inorganic phosphorus had been released.

5 ml of the liquid mixture was neutralized to pH 7 with an aqueous solution of NaOH and passed through an ion-exchange column (Dowex 1, chloride form 10 mm x 150 mm) and eluted with a linear gradient of hydrochloric acid (0-0.7 N HCl). Aliquots of eluted fractions were completely hydrolyzed in order to determine the contents of phosphorus and inositol. The amount of phosphorus versus eluted volume is shown in fig 10. The fraction with the ratio of phosphorus to inositol of three to one was collected. The H-NMR spectrum indicated a substantial number of isomeric products.

Example 20

Chemical synthesis of inositolphosphates.

Polyphosphoric acid (80 % P_2O_5, 3.5 g) was introduced into a glass-stoppered flask and heated to 150°C. Myo-inositol, 0.2 g, was added and the mixture maintained at said temperature for 2 hours until it was neutralized to pH 7 with an aqueous solution of NaOH. The composition obtained was precipitated as a bariumsalt after addition of 10 % excess of 0.1 M bariumacetate solution.

20 mg of the bariumsalt was converted to the acid form by addition of diluted hydrochloric acid and was analyzed by HPLC. The analysis method was calibrated with well-defined inositolphosphates. Fig 11 shows the chromatogram. The fraction determined to be inositoltriphosphate was collected.

The H-NMR spectrum indicated a substantial number of isomeric products.

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Example 21

1.0 kg of rice bran, containing ca 1 % inositolhexaphosphate (IP_6) was suspended in 10 l sodiumacetate buffer at pH 5 at 25°C. After 4 hours when 50 % inorganic phosphorus had been released the slurry was extracted with an addition of 1 l 2 M HCl. The suspension was shaken for 1 hour and subsequently centrifuged. The supernatant was neutralized to pH 7 with an aqueous solution of $Ca(OH)_2$. A precipitate was obtained when 5 l ethanol was added. The calciumsalt consisting of a composition of different inositolphosphates was centrifuged, dried and recrystalized. 20 mg of the recrystallized calciumsalt was converted to the acid form by addition of diluted hydrochloric acid and was analyzed by HPLC. The composition consisted of 40 % inositoltriphosphate of which 70 % was D-myo-inositol-1.2.6-triphosphate. The rest consisted of other inositolphosphates.

Example 22

Characterization of different salts of D-myo-inositol-1.2.6-triphosphate.

70 ml of the fraction with the phosphorus/inositol ratio of three to one obtained in Example 15 was divided in 7 portions. After pH adjustment with 0.1 M NaOH different positive ions in the chloride form was added, one ion to each portion. The salts used were $FeCl_3$, $MgCl_2$, $AlCl_3$, KCl, NH_4Cl, $(CH_3CH_2CH_2CH_2)_4N\ Cl$ and $C_6H_{13}NH_3Cl$ respectively.

After addition of 10 ml ethanol precipitates were formed. The salts were recrystalized and analyzed for the content of phosphorus, carbon, oxygen and metal after recrystallization. Table 1 below shows the composition of purified salts.

Example 23

The effect of IP_3 on platelet aggregation after smoking in humans was studied.

Four young healthy male non-smokers recieved, on two occasions, a capsule containing 50 mg of IP_3 or 50 mg of a placebo. The IP_3 used was the Ca-salt of D-myo-inositol-1.2.6-triphosphate. Neither subject nor investigator knew whether the subject had recieveo IP_3 or placebo.

Two hours after ingestion of the capsule, a blood sample was obtained. The subject then smoked two cigaretts in rapid succession. A second blood sample was obtained after smoking. The aggregation responses of the platelets to ADP and collagen in the two samples were determined, using essentially the same procedure as in Example 1. The results are expressed as change in aggregation from the pre-smoking to the post-smoking sample. A positive sign indicates that aggregation was stronger after smoking.

Aggregating agent	Concentration of aggregating agent	IP_3	Placebo	Difference between IP_3 and placebo
ADP	0.5 mmol	+ 1.5	+ 7.25	5.85
-"-	1 mmol	- 1.5	+ 0.25	1.75
-"-	2.5 mmol	- 1.5	0	1.5
-"-	5 mmol	- 2.5	- 0.75	1.75
Collagen	0.5 mg	+ 5.15	+ 12.25	6.5
-"-	1 mg	- 8.25	+ 1.75	10.0
-"-	2.5 mg	- 3.75	0	3.75
-"-	5 mg	- 1.5	- 0.25	1.25

In the placebo group, smoking caused an increase in aggregation, which was most marked at low concentrations of aggregation agents. In all cases this effect was counteracted by IP_3. Thus IP_3 prevents increase of platelet aggregation caused by smoking.

Example 24

Mice, 10 in each group, were injected intraperitoneally with IP_3 (Na-salt of D-myo-inositol-1.2.6-triphosphate) in three dose levels or with physiological saline. 30 minutes after this injection, all mice except one control group received an intravenous injection of alloxan, 50 mg/kg in saline.

The animals were starved for 12 hours before, and one hour after the alloxan injection. 72 hours after the alloxan injection, a blood sample from the mice were analyzed with respect to glucose level. The results were as follows:

Dose of IP_3 mg/kg	Dose of alloxan mg/kg	Blood glucose
0	0	216
0	50	864
800	50	857
1600	50	677

Alloxan causes diabetes and increased blood glucose level by promotion free radical reactions in the insulin producing cells. With IP_3 there was a dose-dependent decrease in blood glucose levels, and the highest dose gave some protection to the alloxan.

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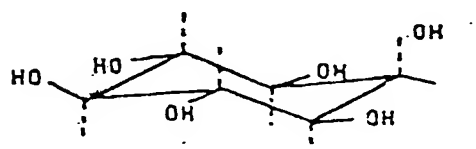
Table 1 Chemical formulations of different salts of IP_3.

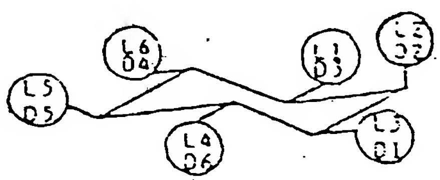
Compound		Elementary analysis					Formula
		Me %	C %	O %	P %	N %	
Calciumsalt of 1.2.6-IP_3	Ca:	22.0	14.1	44.2	17.1	--	Ca_3IP_3
Calciumsalt of 1.3.4-IP_3	Ca:	21.4	13.8	42.9	16.3	--	Ca_3IP_3
Calciumsalt of 1.2.3-IP_3	Ca:	22.7	12.9	45.6	18.2	--	Ca_3IP_3
Calciumsalt of 1.2.5-IP_3	Ca:	23.2	13.3	44.7	16.9	--	Ca_3IP_3
Zincsalt of 1.2.6-IP_3	Zn:	31.5	12.1	38.2	14.7	--	Zn_3IP_3
Sodiumsalt of 1.2.6-IP_3	Na:	23.1	12.8	44.6	15.8	--	Na_6IP_3
Ironsalt of 1.2.6-IP_3	Fe:	21.5	13.1	43.4	18.2	--	Fe_2IP_3
Potassium, magnesiumsalt of 1.2.6-IP_3	K: Mg:	27.5 3.8	11.3	38.7	14.1	--	$K_4Mg\ IP_3$
Magnesiumsalt of 1.2.6-IP_3	Mg:	15.8	16.0	50.8	18.9	--	Mg_3IP_3
Aluminiumsalt of 1.2.6-IP_3	Al:	12.6	15.3	53.3	21.2	--	Al_2IP_3
Ammoniumsalt of 1.2.6-IP_3		--	13.1	47.8	16.9	15.6	$(NH_4)_6IP_3$
Tetrabutyl ammoniumsalt of 1.2.6-IP_3		--	57.0	20.1	7.8	3.5	$[(CH_3(CH_2)_3)_4N]_3H_3IP_3$
Cyclohexylammoniumsalt of 1.2.6-IP_3		--	39.5	35.1	12.6	6.2	$[C_6H_3NH_3]_3H_3IP_3$

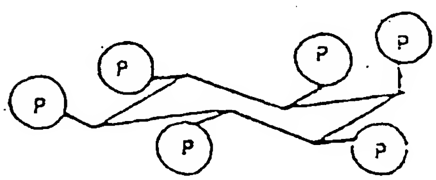
For purposes of further understanding the invention, formulas are given below of the IP_3 isomers of the invention. Formulas are also given for IP_6, IP_5, IP_4 and IP_2.

The lower phosphate-esters of myoinositol are named depending on where the phosphoric acid groups are situated on the inositol ring, with the numbering giving as low position numbers as possible. L and D stand for clockwise and counterclock-wise counting respectively, and are used depending on whith result gives the lowest position number. The carbon atom which has an axial phosphoric acid group always has the position number 2. The structural formulae below are simplified to the acid form.

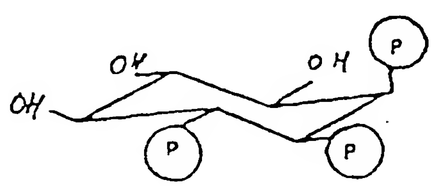


myo-inositol; C_6H_6 $(OH)_6$

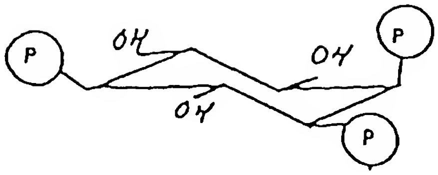




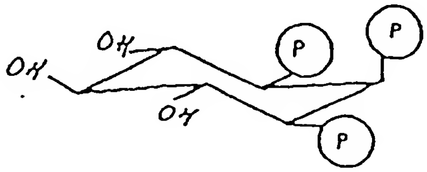
1.2.3.4.5.6-hexakis-(dihydrogenphosphate)-myo-inositol alternatively myo-inositol hexakis (phosphate) or IP_6



D-myo-inositol-1,2,6-triphosphate alternatively D-1,2,6-IP_3

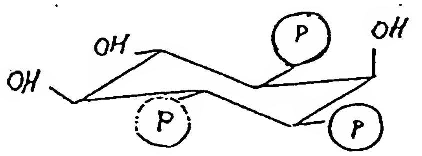


D-myo-inositol-1,2,5-triphosphate alternatively D-1,2,5-IP_3

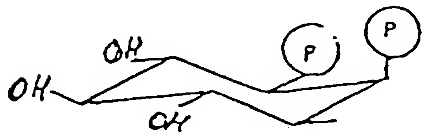


myo-inositol-1,2,3-triphosphate alternatively 1,2,3-IP_3

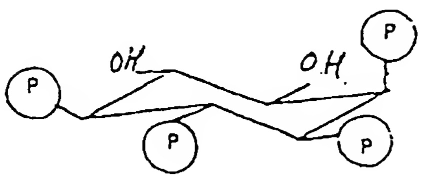
P = $O-PO_3H_2$



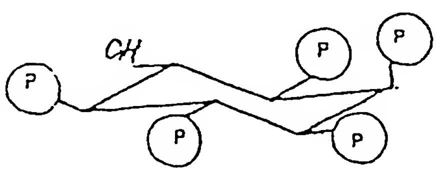
L-myo-inositol-1.3.4-triphosphate alternatively L-1.3.4-IP_3



L-myo-inositol-1,2-diphosphate alternatively L-1,2-IP_2



D-myo-inositol-1,2,5,6-tetra-phosphate or D-1,2,5,6-IP_4



L-myo-inositol-1,2,3,4,5-penta phosphate or L-1,2,3,4,5-IP_5

P = $-O-PO_3H_2$

Claims

1. An inositoltriphosphate (IP_3) selected from the group consisting of D-myo-inositol-1.2.6-triphosphate, D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate in either acid or salt form.

2. An IP_3 according to claim 1, which consists of D-myo-inositol-1.2.6-triphosphate.

3. An IP_3 according to claim 1, which consists of myo-inositol-1.2.3-triphosphate.

4. An IP_3 according to claim 1, in substantially pure form.

5. An IP_3 in acid form according to claim 1 in the form of an aqueous solution.

6. An IP_3 in acid form according to claim 1, in the form of an aqueous solution wherein the concentration of acid is from 10-45% by weight based on the total weight of the solution.

7. An IP_3 in salt form according to claim 1 the cations of which are selected from the group consisting of alkali metals and alkaline earth metals.

8. A salt according to claim 7, wherein the cations are selected from the group consisting of lithium, sodium, potassium, calcium or magnesium.

9. An IP_3 in salt form according to claim 1, wherein the cations are selected from the group consisting of aluminium, zinc or iron.

10. An IP_3 in salt form according to claim 1, wherein the salt is an ammonium salt or an organic amine salt.

11. A method of producing an inositol-triphosphate (IP_3) selected from the group consisting of D-myo-inositol-1.2.6-triphosphate, D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate in either acid or salt form, wherein an inositol-hexaphosphate containing material is incubated at a temperature ranging between 20 and 70°C and at a pH of 4 to 8 with phytase until the liberation of about 30-60 % of the total ester phosphorous is achieved, hereafter separating by chromatography the mixture obtained to isolate the IP_3-containing fracion, optionally subjecting said fraction to further chromatographic separation and isolating specific isomeric IP_3 in acid or salt form in solution.

12. A method according to claim 11 wherein the phytase is a yeast phytase.

13. A method according to claim 12, wherein the yeast is baker's yeast.

14. A method of producing D-myo-inositol-1.2.6- triphosphate in either acid or salt form, wherein an inositol-hexaphosphate containing material is incubated at a temperature ranging between 20 and 70°C with yeast phytase until the liberation of about 30-60 % of the total ester phosphorous is achieved, hereafter separating by chromatography the mixture obtained to isolate the IP_3-containing fraction and isolating D-myo-inositol-1.2.6-triphosphate in acid or salt form in solution.

15. A method according to claim 14, wherein the yeast is baker's yeast.

16. An inositol-phosphate composition comprised of D-myo-inositol-1.2.6-triphosphate and at least one of D-myo-inositol-1.2.5-triphosphate, L-myo-inositol-1.3.4-triphosphate and myo-inositol-1.2.3-triphosphate in acid or salt form, characterized in that it contains 20-99.5% by weight of said IP_3 and the content of D-myo-inositol-1,2.6-triphosphate is in the range from 50-100% by weight based on the total content of said inositoltriphosphates, the balance including inositol-phosphates other than IP_3.

17. A composition according to claim 16, wherein the IP_3-fraction is present in an amount of 30-99.5% by weight.

18. A composition according to claim 16, wherein the IP_3 is essentially D-myo-inositol-1.2.6-triphosphate.

Figure 1: Hydrolysis of sodium phytate with wheat phytase.

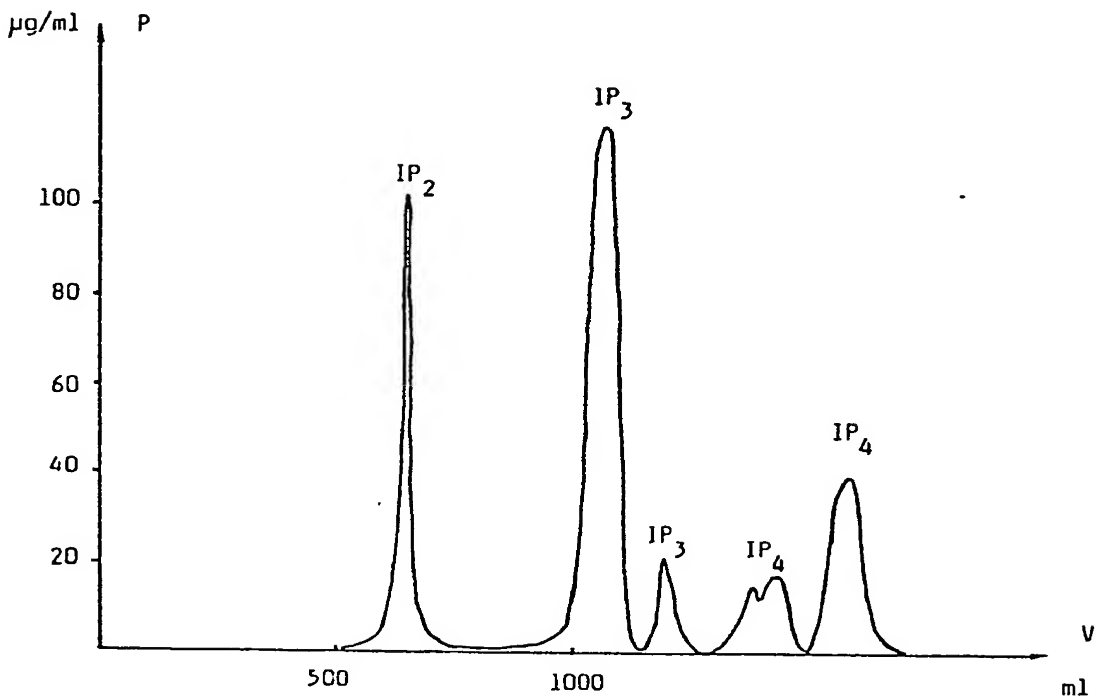


Figure 2: Separation of isomers of inositoltriphosphates.

a = myo-inositol-1.3.4-triphosphate

b = myo-inositol-1.2.3-triphosphate

c = myo-inositol-1.2.6-triphosphate

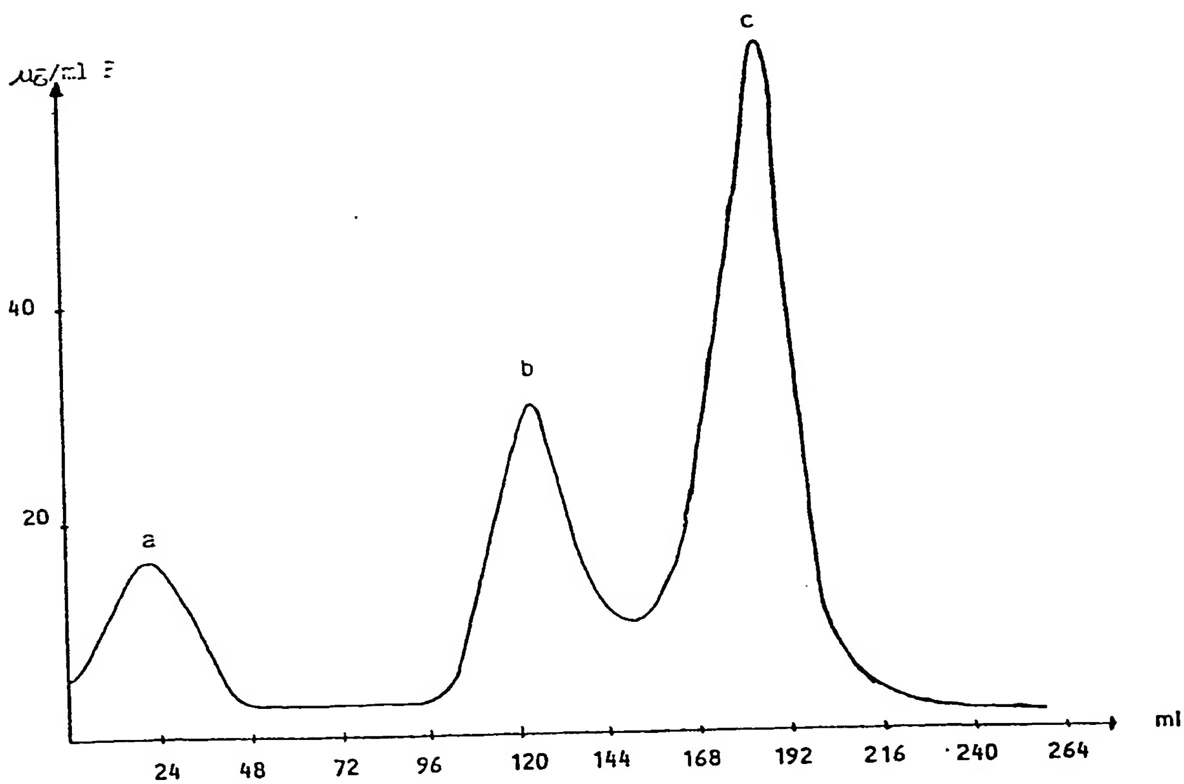


Figure 3a: H-NM[illegible]ectrum of myo-inositol-1.2.6-triphos[illegible]e.

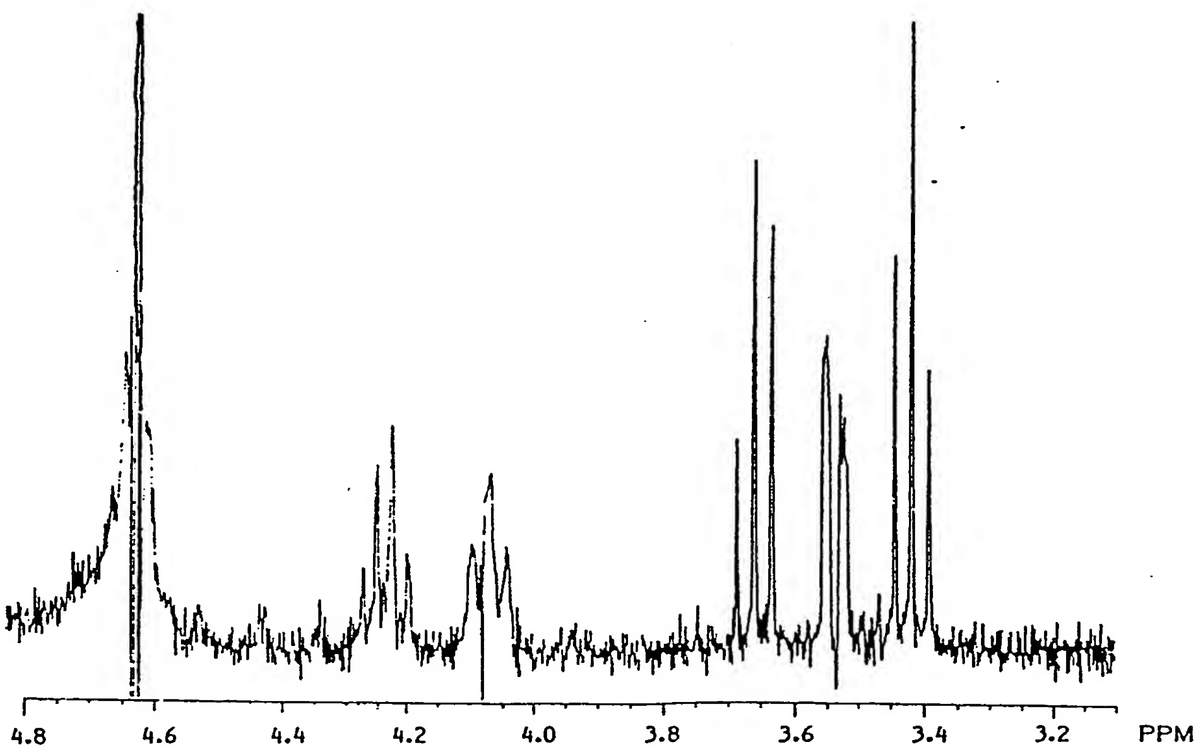


Figure 3b: H-NMR-spectrum of myo-inositol-1.2.3-triphosphate

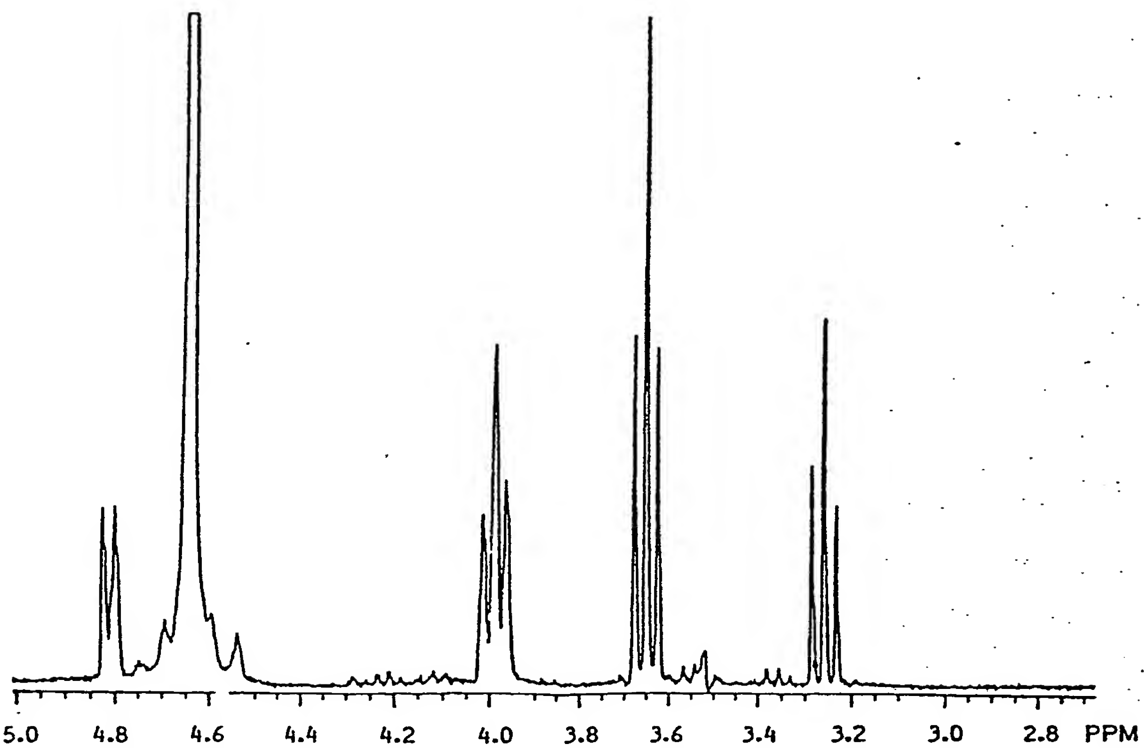


Figure 3c: H-NMR-spectrum of myo-inositol-1.3.4-triphosphate.

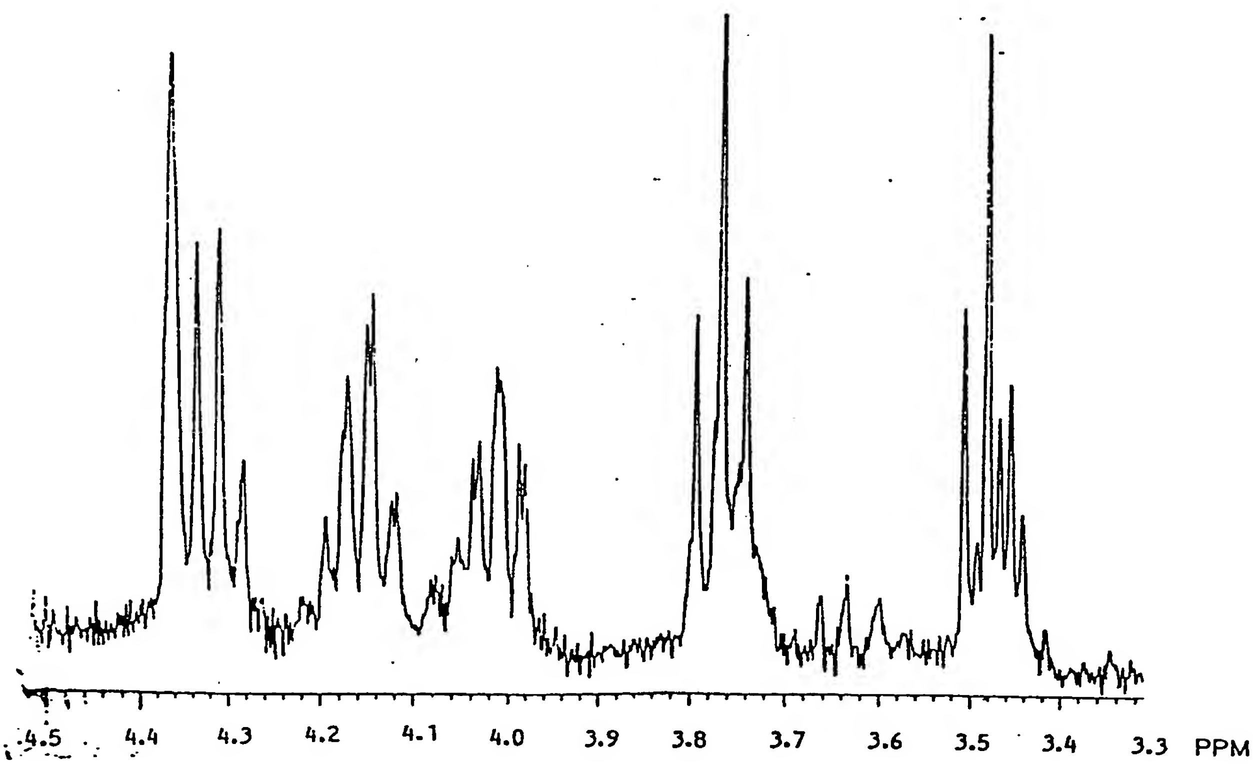


Figure 4: H-NMR-spectrum of myo-inositol-1.2.5-triphosphate

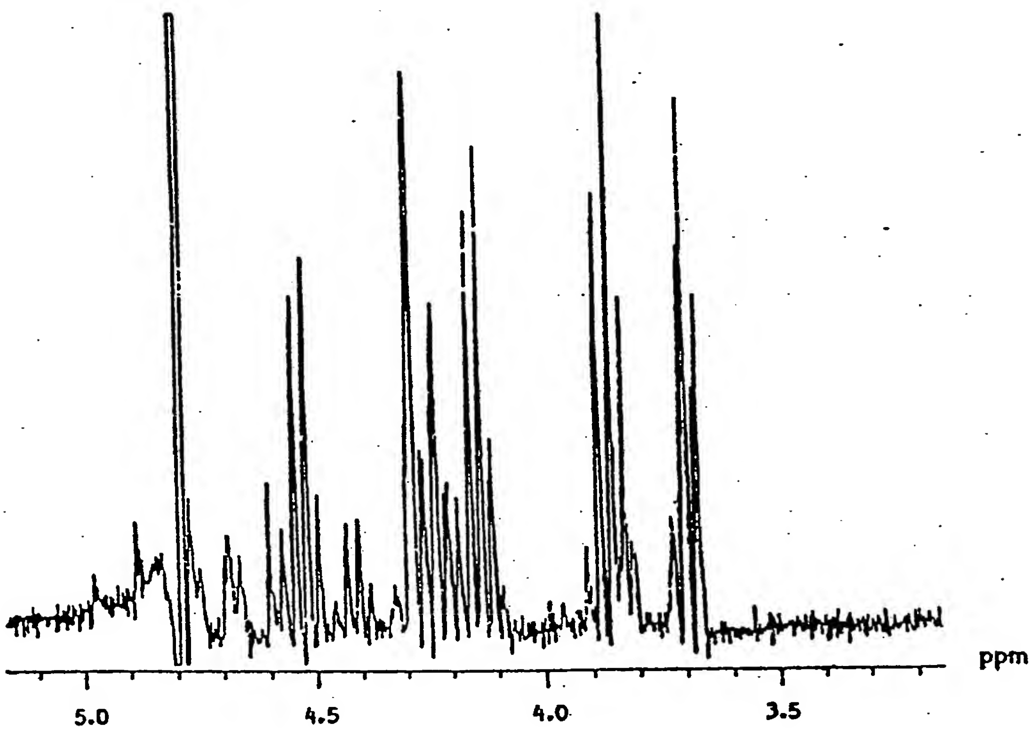


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Figure 5: Optical resolution of myo-inositol-1.2.6-triphosphate and myo-inositol-1.3.4-triphosphate.

a = D-myo-inositol-1.2.6-triphosphate

b = L-myo-inositol-1.3.4-triphosphate

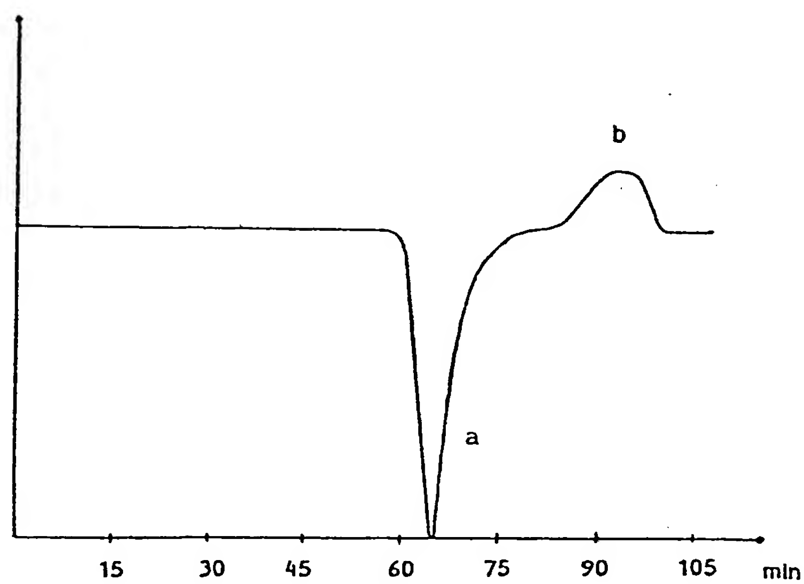

Figure 6: Titration of IP_3 with NaOH.

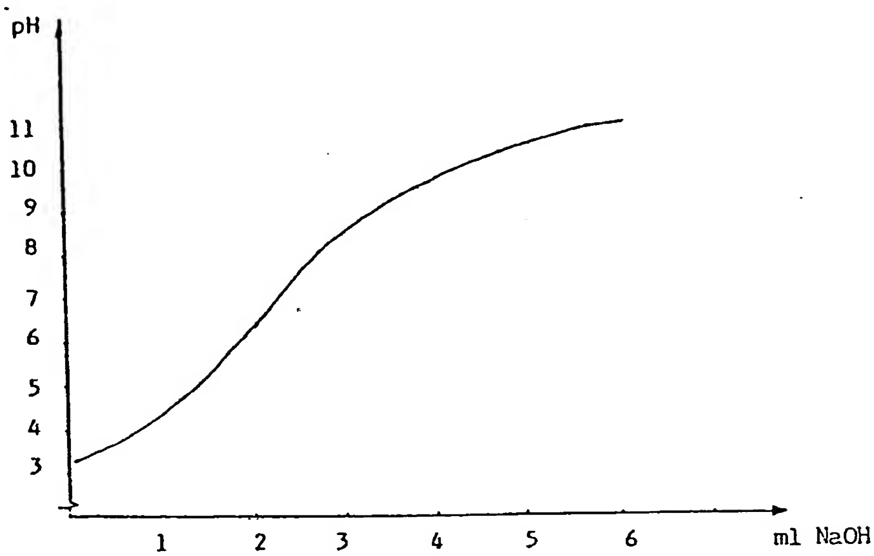

Figure 7: Titration of IP_3 with NaOH in the presence of metal ions

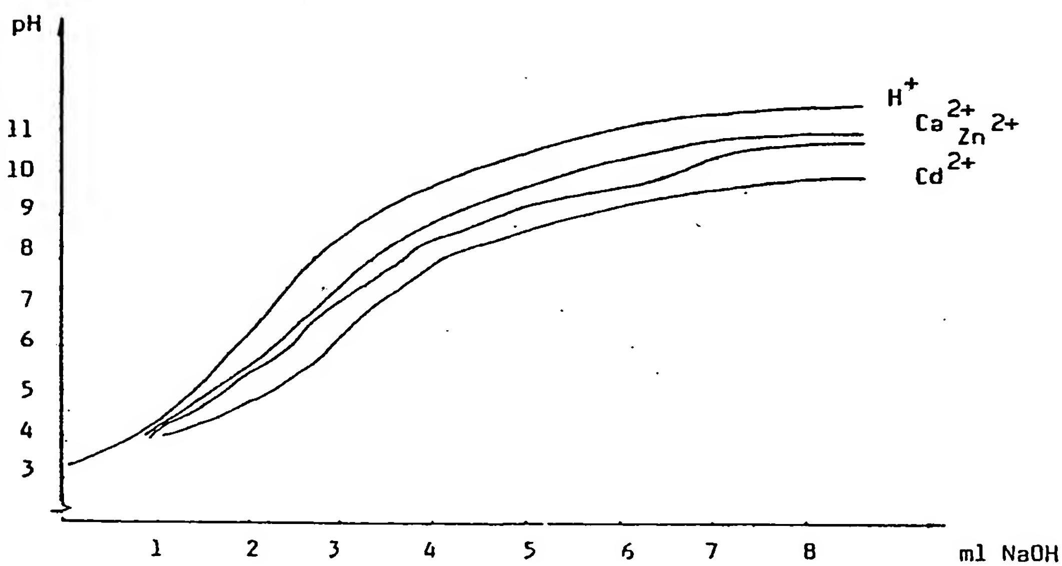

Figure 8 Hydrolysis of sodiumphytate with baker's yeast.

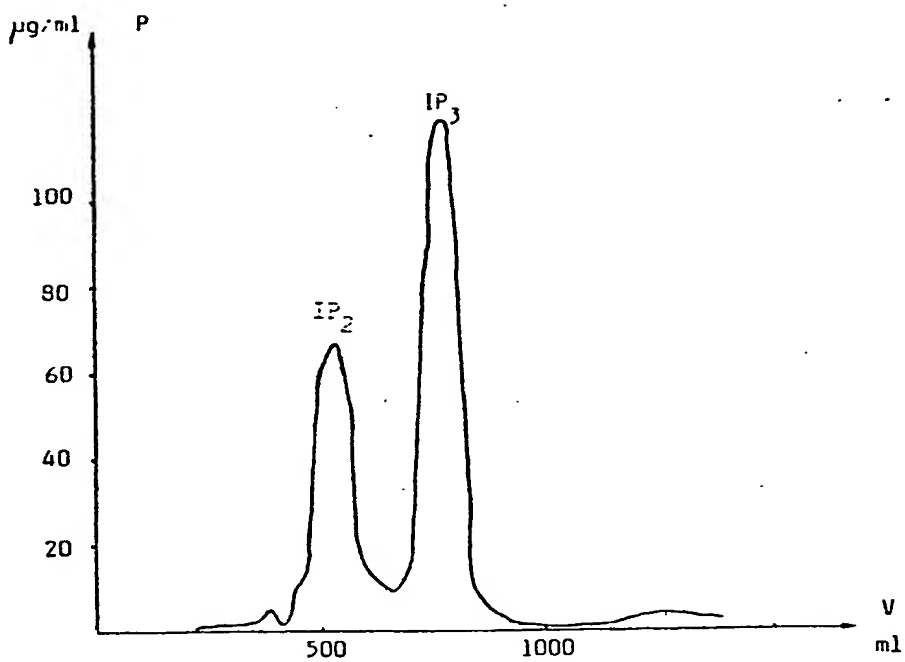

Figure 9 Optical resolution of myo-inositol-1.2.6-triphosphate.

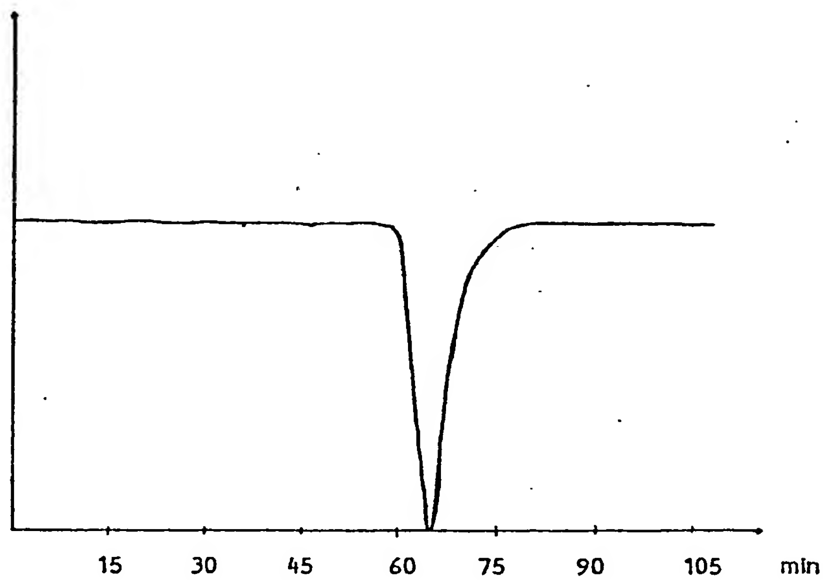

Figure 10 Chemical hydrolysis of sodiumphytate.

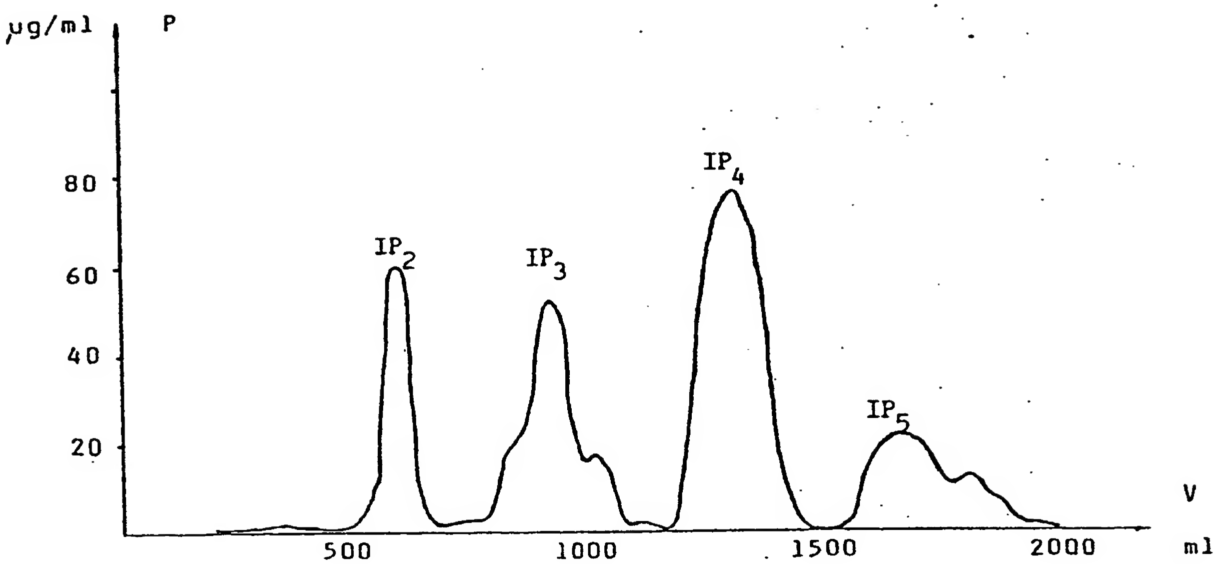

Figure 11 HPLC-chromatogram of chemically synthesized inositol phosphates.

a = IP_2
b = IP_3
c = IP_4
d = IP_5
e = IP_6

European Patent Office

EUROPEAN SEARCH REPORT

0179440
Application number

EP 85 11 3382

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 56, 1962, no. 15807f, Columbus, Ohio, US; R.V. TOMLINSON et al.: "Myoinositol polyphosphate intermediates in the dephosphorylation of phytic acid by phytase", & BIOCHEMISTRY 1, 166-71(1962) * Abstract * ---	1-18	C 07 F 9/117 C 12 P 9/00
X	CHEMICAL ABSTRACTS, vol. 78, 1973, no. 155960x, Columbus, Ohio, US; P.E. LIM et al.: "Phytases. II. Properties of phytase fractions F1 and F2 from wheat bran and the myo-inositol phosphates produced by fraction F2", & BIOCHIM. BIOPHYS. ACTA 1973 302(2), 316-28 * Abstract * ---	1-18	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 101, 1984, page 128, no. 223262s, Columbus, Ohio, US; R.F. IRVINE et al.: "Inositol trisphosphates in carbachol-stimulated rat parotid glands", & BIOCHEM. J. 1984 223(1), 237-43 * Abstract * -----	1-18	C 07 F 9/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	07-01-1986	BESLIER L.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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P : intermediate document

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E : earlier patent document, but published on, or after the filing date
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